Application

for

United States Letters Patent

To all whom it may concern:

  Be it known that Graham P. Allaway, Virginia M. Litwin,
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have invented certain new and useful improvements in

A METHOD FOR PREVENTING HIV-1 INFECTION OF CD4+ CELLS

of which the following is a full, clear and exact description.

A METHOD FOR PREVENTING HIV-1 INFECTION OF CD4⁺ CELLS

This application claims priority of U.S. Provisional Application No. 60/019,715, filed June 14, 1996, and U.S. Provisional Application No. 60/014,532, filed April 2, 1996, the content of which are incorporated by reference into this
5   application.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into
10   this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each series of experiments.

15   **Background of the Invention**
Chemokines are a family of related soluble proteins of molecular weight between 8 and 10KDa, secreted by lymphocytes and other cells, which bind receptors on target cell surfaces resulting in the activation and mobilization
20   of leukocytes, for example in the inflammatory process. Recently, Cocchi et al. demonstrated that the chemokines RANTES, MIP-1α and MIP-1β are factors produced by CD8⁻ T lymphocytes which inhibit infection by macrophage-tropic primary isolates of HIV-1, but not infection by
25   laboratory-adapted strains of the virus (1). These chemokines are members of the C-C group of chemokines, so named because they have adjacent cysteine residues, unlike the C-X-C group which has a single amino acid separating these residues (2). While Cocchi et al. found that
30   expression of HIV-1 RNA was suppressed by treatment with the chemokines, they did not identify the site of action of these molecules.
A resonance energy transfer (RET) assay of HIV-1 envelope

glycoprotein-mediated membrane fusion was used to determine
whether fusion mediated by the envelope glycoprotein from
the primary macrophage-tropic isolate of HIV-1$_{JR-FL}$ would be
specifically inhibited by chemokines, when compared with
5   fusion mediated by the envelope glycoprotein from the
laboratory-adapted T lymphotropic strain HIV-1$_{LAI}$. As
described below, it was demonstrated that this is indeed the
case. This demonstrates that some chemokine receptors are
fusion accessory molecules required for HIV-1 infection.
10   Previous studies have indicated that unidentified cell
surface molecules are required for virus entry in addition
to the HIV-1 receptor, CD4. While CD4 is required for HIV-1
attachment, the accessory molecules are required for the
membrane fusion step of entry. These accessory molecules
15   are generally expressed only on human cells, so HIV-1 does
not infect non-human CD4$^+$ cells (3-6). Moreover it is
possible to complement non-human CD4$^+$ cells by fusing them
(using polyethylene glycol) with CD4$^-$ human cells, resulting
in a heterokaryon which is a competent target for HIV-1
20   envelope-mediated membrane fusion (7,8). These studies have
been performed using laboratory-adapted T lymphotropic
strains of the virus.

In some cases, it appears that fusion accessory molecules
25   are found on a subset of human CD4$^+$ cells and are required
for infection by HIV-1 isolates with particular tropisms.
For example, macrophage-tropic primary strains of HIV-1 such
as HIV-1$_{JR-FL}$ may have different requirements for accessory
molecules compared with laboratory-adapted T lymphotropic
30   strains such as HIV-1$_{LAI}$. This phenomenon may explain
differences in tropism between HIV-1 strains.

The current invention comprises a series of new therapeutics
for HIV-1 infection. It was demonstrated for the first time
35   that chemokines act at the fusion step of HIV-1 entry and

specifically inhibit membrane fusion mediated by the envelope glycoprotein of primary macrophage-tropic primary viral isolates, not laboratory-adapted T lymphotrophic strains of the virus. Primary macrophage-tropic isolates of the virus are of particular importance since they are the strains usually involved in virus transmission, and may have particular importance in the pathogenesis of HIV-1 infection.

These results were obtained using a resonance energy transfer (RET) assay of HIV-1 envelope-mediated membrane fusion. Moreover, this assay is used to identify non-chemokines, including fragments of chemokines and modified chemokines, that inhibit HIV-1 envelope glycoprotein-mediated membrane fusion and thereby neutralize the virus, yet do not induce an inflammatory response.

Summary of the Invention

This invention provides a method for inhibiting fusion of HIV-1 to CD4⁺ cells which comprises contacting CD4⁺ cells with a non-chemokine agent capable of binding to a chemokine
5   receptor in an amount and under conditions such that fusion of HIV-1 to the CD4⁺ cells is inhibited.

This invention also provides a method for inhibiting HIV-1 infection of CD4⁺ cells which comprises contacting CD4⁺ cells
10 with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4⁺ cells is inhibited, thereby inhibiting the HIV-1 infection.

15 This invention further provides non-chemokine agents capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4⁺ cells.

This invention provides an agent which is capable of binding
20 to fusin and inhibiting infection. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is an polypeptide. In still another embodiment, the agent is an antibody or a portion of an antibody. In a separate embodiment, the agent is a nonypeptidyl agent.
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In   addition,   this   invention   provides   pharmaceutical compositions comprising an amount of the above non-chemokine agents or agents capable of binding to fusin effective to inhibit fusion of HIV-1 to CD4⁺ cells and a pharmaceutically
30 acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4⁺ cells comprising a non-chemokine agent linked
35 to a ligand capable of binding to a cell surface receptor of

the CD4$^+$ cells other than the chemokine receptor such that
the binding of the non-chemokine agent to the chemokine
receptor does not prevent the binding of the ligand to the
other receptor.

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This invention also provides a pharmaceutical composition
comprising an amount of the above-described composition of
matter effective to inhibit fusion of HIV-1 to CD4$^-$ cells
and a pharmaceutically acceptable carrier.

10

This invention provides a composition of matter capable of
binding to the chemokine receptor and inhibiting fusion of
HIV-1 to CD4$^+$ cells comprising a non-chemokine agent linked
to a compound capable of increasing the *in vivo* half-life of

15   the non-chemokine agent.

This invention also provides a pharmaceutical composition
comprising an amount of a composition of matter comprising
a non-chemokine agent linked to a compound capable of

20   increasing the *in vivo* half-life of the non-chemokine agent
effective to inhibit fusion of HIV-1 to CD4$^+$ cells and a
pharmaceutically acceptable carrier.

This invention provide methods for reducing the likelihood

25   of HIV-1 infection in a subject comprising administering an
above-described pharmaceutical composition to the subject.
This invention also provides methods for treating HIV-1
infection in a subject comprising administering an above-
described pharmaceutical composition to the subject.

30

This invention also provides methods for determining whether
a non-chemokine agent is capable of inhibiting the fusion of
HIV-1 to a CD4$^+$ cell which comprise: (a) contacting (i) a
CD4$^+$ cell which is labeled with a first dye and (ii) a cell

35   expressing the HIV-1 envelope glycoprotein on its surface

which is labeled with a second dye, in the presence of an excess of the agent under conditions permitting the fusion of the CD4$^+$ cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of the agent, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and (c) determining whether there is a reduction of resonance energy transfer, when compared with the resonance energy transfer in the absence of the agent, a decrease in transfer indicating that the agent is capable of inhibiting fusion of HIV-1 to CD4$^+$ cells.

Brief Description of the Figures

Figure 1. **Membrane fusion mediated by the HIV-1$_{JR-FL}$ envelope glycoprotein is inhibited by RANTES, MIP-1α and MIP-1β.**

%RET resulting from the fusion of PM1 cells and HeLa-env$_{JR-FL}$ (■) or HeLa-env$_{LAI}$ (♦) was measured in the presence and absence of recombinant human chemokines at a range of concentrations: RANTES (80 - 2.5 ng/ml), MIP-1α (400 - 12.5 ng/ml) and MIP-1β (200 - 6.25 ng/ml), as indicated. Chemokines were added simultaneously with the cells at the initiation of a four hour incubation. Data are representative of more than three independent experiments which were run in duplicate. The percent inhibition of RET is defined as follows:

% Inhibition = 100 . [(Max RET - Min RET) - (Exp RET- Min RET)]/(Max RET - Min RET)

where Max RET is the %RET value obtained at four hours with HeLa-env cells and CD4-expressing cells in the absence of an inhibitory compound; Exp RET is the %RET value obtained for the same cell combination in the presence of an inhibitory compound and Min RET is the background %RET value obtained using HeLa cells in place of HeLa envelope-expressing cells.

Figure 2. **CD4:HIV-1 gp120 binding in the presence of human chemokines.**

The binding of soluble human CD4 to HIV-1$_{LAI}$ and

HIV-1$_{JR-FL}$ gp120 was determined in an ELISA assay in the presence and absence of the monoclonal antibody OKT4A or recombinant human chemokines at a range of concentrations, identical to those used in the RET inhibition studies of Figure 1: OKT4A (62 - 0.3 nM), RANTES (10.3 - 0.3 nM), MIP-1α (53.3 - 2.9 nM), and MIP-1β (25.6 - 0.8 nM). Inhibitors were added simultaneously with biotinylated HIV-1 gp120 to soluble CD4 coated microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). Following a two hour incubation at room temperature and extensive washing, an incubation with streptavidin-horseradish peroxidase was performed for one hour at room temperature. Following additional washes, substrate was added and the OD at 492 nm determined in an ELISA plate reader. Data are representative of two independent experiments which were run in quadruplicate.

Figure 3. **Specificity. time course and stage of ß-chemokine inhibition of HIV-1 replication.**

(a) PM1 cells (1 x10^6) were preincubated with RANTES + MIP- 1α + MIP-1ß (R/Mα/Mß; 100ng/ml of each) for 24h (-24h) or 2h (-2h), then washed twice with phosphate buffered saline (PBS). HIV-1 (BaL env-complemented) virus (50ng of p24; see legend to Table 1) was added for 2h, then the cells were washed and incubated for 48h before measurement of luciferase activity in cell lysates as described previously (10,11). Alternatively, virus and R/Mα/Mß were added simultaneously to cells, and at the indicated time points (1h, 3h, etc) the cells were washed

twice in PBS, resuspended in culture medium and incubated for 48h prior to luciferase assay. Time 0 represents the positive control, to which no ß-chemokines were added. +2h represents the mixture of virus with cells for 2h prior to washing twice in PBS, addition of R/Mα/Mß and continuation of the culture for a further 48h before luciferase assay.

(b) PM1 cells (1×10^6) were infected with HIV-1 (500pg p24) grown in CEM cells (NL4/3; lanes 1-4) or macrophages (ADA; lanes 5-8), in the presence of 500ng/ml of RANTES (lanes 1 and 5) or MIP-1ß (lanes 2 and 6), or with no ß-chemokine (lanes 4 and 8). Lanes 3 and 7 are negative controls (no virus). All viral stocks used for the PCR assay were treated with DNAse for 30 min at 37°C, and tested for DNA contamination before use. After 2h, the cells were washed and resuspended in medium containing the same ß-chemokines for a further 8h. DNA was then extracted from infected cells using a DNA/RNA isolation kit (US Biochemicals). First round nested PCR was performed with primers: U3+, 5'-CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGG-3' (SEQ ID NO:1) preGag, 5'-AGCAAGCCGAGTCCTGCGTCGAGAG-3' (SEQ ID NO:2) and the second round with primers: LTR-test, 5'-GGGACTTTCCGCTGGGGACTTTC 3' (SEQ ID NO :3) LRC2, 5'-CCTGTTCGGGCGCCACTGCTAGAGATTTTCCAC 3' (SEQ ID NO:4) in a Perkin Elmer 2400 cycler with the following amplification cycles: 94°C for 5 min, 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 30s, 72°C for 7 min. M indicates 1kb DNA ladder; 1, 10, 100, 1000 indicate number of reference

plasmid (pAD8) copies. The assay can detect 100
copies of reverse transcripts.

Figure 4: **HIV-1 env-mediated membrane fusion of cells**
5   **transiently expressing C-C CKR-5.**

Membrane fusion mediated by ß-chemokine receptors
expressed in HeLa cells was demonstrated as
follows: Cells were transfected with control
10   plasmid pcDNA3.1 or plasmid pcDNA3.1 -CKR
constructs using lipofectin (Gibco BRL). The
pcDNA3.1 plasmid carries a T7-polymerase promoter
and transient expression of ß-chemokine receptors
was boosted by infecting cells with $1x10^7$ pfu of
15   vaccinia encoding the T7-polymerase (vFT7.3) 4h
post-lipofection (9). Cells were then cultured
overnight in R18-containing media and were tested
for their ability to fuse with HeLa-JR-FL cells
(filled columns) or HeLa-BRU cells (hatched
20   column) in the RET assay. The %RET with control
HeLa cells was between 3% and 4% irrespective of
the transfected plasmid.

Figure 5 **Membrane fusion mediated by the HIV$_{LAI}$ envelope**
25   **glycoprotein is inhibited by SDF-1.**

% RET resulting from the fusion of PM1 cells and
HeLa-env$_{JR-FL}$ or HeLa-env$_{LAI}$ cells (as indicated on
the graph) was measured in the presence of
30   recombinant SDF-1α(Gryphon Science, San
Francisco) at the indicated concentrations.
Experimental method as described in the legend to
Fig. 1.

Detailed Description of the Invention

This invention provides a method for inhibiting fusion of HIV-1 to CD4+ cells which comprises contacting CD4+ cells with a non-chemokine agent capable of binding to a chemokine
5 receptor in an amount and under conditions such that fusion of HIV-1 to the CD4+ cells is inhibited.

This invention also provides a method for inhibiting HIV-1 infection of CD4+ cells which comprises contacting CD4+ cells
10 with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4+ cells is inhibited, thereby inhibiting the HIV-1 infection.

15 In this invention, a chemokine means RANTES, MIP-1-α, MIP-1-β or another chemokine which blocks HIV-1 infection. A chemokine receptor means a receptor capable of binding RANTES, MIP-1-α, MIP-1-β or another chemokine which blocks HIV-1 infection.
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Throughout this application, the receptor "fusin" is also named CXCR4 and the chemokine receptor C-C CKR5 is also named CCR5.

25 The HIV-1 used in this application unless specified will mean clinical or primary or field isolates or HIV-1 viruses which maintain their clinical characteristics. The HIV-1 clinical isolates may be passaged in primary peripheral blood mononuclear cells. The HIV-1 clinical isolates may be
30 macrophage-trophic.

The non-chemokine agents of this invention are capable of binding to chemokine receptors and inhibiting fusion of HIV-1 to CD4+ cells. The non-chemokine agents include, but are
35 not limited to, chemokine fragments and chemokine

derivatives and analogues, but do not include naturally occurring chemokines. The non-chemokine agents include multimeric forms of the chemokine fragments and chemokine derivatives and analogues or fusion molecules which contain chemokine fragments, derivatives and analogues linked to other molecules.

In an embodiment of this invention, the non-chemokine agent is an oligopeptide. In another embodiment, the non-chemokine agent is a polypeptide. In still another embodiment, the non-chemokine agent is an antibody or a portion thereof. Antibodies against the chemokine receptor may easily be generated by routine experiments. It is also within the level of ordinary skill to synthesize fragments of the antibody capable of binding to the chemokine receptor. In a further embodiment, the non-chemokine agent is a nonpeptidyl agent.

Non-chemokine agents which are purely peptidyl in composition can be either chemically synthesized by solid-phase methods (Merrifield, 1966) or produced using recombinant technology in either prokaryotic or eukaryotic systems. The synthetic and recombinant methods are well known in the art.

Non-chemokine agents which contain biotin or other nonpeptidyl groups can be prepared by chemical modification of synthetic or recombinant chemokines or non-chemokine agents. One chemical modification method involves periodate oxidation of the 2-amino alcohol present on chemokines or non-chemokine agents possessing serine or threonine as their N-terminal amino acid (Geophegan and Stroh, 1992). The resulting aldehyde group can be used to link peptidyl or non-peptidyl groups to the oxidized chemokine or non-chemokine agent by reductive amination, hydrazine, or other

chemistries well known to those skilled in the art.

As used herein, a N-terminus of a protein should mean the terminus of the protein after it has been processed. In
5   case of a secretory protein which contains a cleavable signal sequence, the N-terminus of a secretory protein should be the terminus after the cleavage of a signal peptide.

10   This invention provides a method of identifying these non-chemokine agents. One way of identifying such agents, including non-peptidyl agents, that bind to a chemokine receptor and inhibit fusion of HIV-1 to CD4$^+$ cells is to use the following assay: 1) Incubate soluble CD4 with
15   biotinylated gp120 from HIV-1$_{JR-FL}$ or HIV-1$_{LAI}$; 2) Incubate this complex with CCR5 or CXCR4-expressing cells (for HIV-1$_{JR-FL}$ or HIV-1$_{LAI}$ gp120s, respectively) that do not express CD4, in the presence of absence of a candidate inhibitor; 3) Wash and then incubate with streptavidin-phycoerythrin; and
20   4) Wash and then measure the amount of bound gp120 using a flow cytometer or fluorometer and calculate the degree of inhibition of binding by the inhibitor.

Alternative methods to detect bound gp120 can also be used
25   in place of the biotinylated gp120-streptavidin-phycoerythrin method described above. For example, peroxidase-conjugated gp120 could be used in place of the biotinylated gp120 and binding detected using an appropriate colorimetric substrate for peroxidase, with a spectrometric
30   readout.

This invention further provides the non-chemokine agents identified by the above methods.

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This invention provides a non-chemokine agent capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4⁺ cells. In an embodiment, the non-chemokine is a polypeptide. In a further embodiment, this polypeptide is a fragment of the chemokine RANTES (Gong et al., 1996). In a still further embodiment, the polypeptide may also comprise the RANTES sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first eight N-terminal amino acids of the RANTES sequence (SEQ ID NO:5).

In a separate embodiment, the polypeptide may comprise the MIP-1ß sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first seven, eight, nine or ten N-terminal amino acids of the MIP-1ß sequence.

In another embodiment of non-chemokine agent, the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by addition of an amino acid or oligopeptide. In a separate embodiment, the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by removing the N-terminal alanine and replaced it by serine or threonine and additional amino acid or oligopeptide or nonpeptidyl moiety. In a further embodiment, the additional amino acid is methionine.

As described _infra_ in the section of Experimental Details, a cofactor for HIV-1 fusion and entry was identified and designated "fusin" (Feng et al., 1996). This invention provides an agent which is capable of binding to fusin and inhibiting infection. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is an polypeptide.

In a further embodiment, the polypeptide comprises SDF-1 with deletion of the N-terminal amino acids of said sequence. The deletion may be the first six, seven, eight, or nine N-terminal amino acids of the SDF-1 sequence.

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This invention also provides the above non-chemokine agent, wherein the polypeptide comprises SDF-1 sequence with the N-terminal sequence modified to produce antagonistic effect to SDF-1. One modification is to replace the N-terminal

10   glycine of SDF-1 by serine and derivatized with biotin. Another modification is to replace the N-terminal glycine of SDF-1 by serine and derivatized with methionine. A further modification is to add the N-terminus of SDF-1 with a methionine before the terminal glycine.

15

In still another embodiment, the agent is an antibody or a portion of an antibody. In a separate embodiment, the agent is a nonpeptidyl agent.

20   The agents capable of binding to fusin may be identified by screening different compounds for their capability to bind to fusin in vitro.

A suitable method has been described by Fowlkes, et al.

25   (1994), international application number: PCT/US94/03143, international publication number: WO 94/23025, the content of which is incorporated by reference into this application. Briefly, yeast cells having a pheromone system are engineered to express a heterologous surrogate of a yeast

30   pheromone system protein. The surrogate incorporates fusin and under some conditions performs in the pheromone system of the yeast cell a function naturally performed by the corresponding yeast pheromone system protein. Such yeast cells are also engineered to express a library of peptides

35   whereby a yeast cell containing a peptide which binds fusin

exhibits modulation of the interaction of surrogate yeast pheromone system protein with the yeast pheromone system and this modulation is a selectable or screenable event. Similar approaches may be used to identify agents capable of binding to both fusin and the chemokine receptor C-C CKR-5.

This invention also provides pharmaceutical compositions comprising an amount of such non-chemokine agents or agents capable of binding to fusin effective to inhibit fusion of HIV-1 to CD4⁺ cells and a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers are well known to those skilled in the art. Such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4⁺ cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the CD4⁺ cells other than the chemokine receptor such that

the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the other receptor. In an embodiment, the cell surface receptor is CD4. In another embodiment, the ligand is an antibody or a portion of an antibody.

This invention also provides a pharmaceutical composition comprising an amount of an above-described composition of matter effective to inhibit fusion of HIV-1 to CD4$^+$ cells and a pharmaceutically acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4$^+$ cells comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent. In an embodiment, the compound is polyethylene glycol.

This invention also provides a pharmaceutical composition comprising an amount of a composition of matter comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent effective to inhibit fusion of HIV-1 to CD4$^+$ cells and a pharmaceutically acceptable carrier.

This invention provide methods for reducing likelihood of HIV-1 infection in a subject comprising administering the above-described pharmaceutical compositions to the subject. This invention also provides methods for treating HIV-1 infection in a subject comprising administering the above-described pharmaceutical compositions to the subject.

This invention also provides methods for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a CD4$^+$ cell which comprise: (a) contacting (i) a

CD4$^+$ cell which is labeled with a first dye and (ii) a cell expressing the HIV-1 envelope glycoprotein on its surface which is labeled with a second dye, in the presence of an excess of the agent under conditions permitting the fusion of the CD4$^+$ cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of the agent, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and (c) determining whether there is a reduction of resonance energy transfer, when compared with the resonance energy transfer in the absence of the agent, a decrease in transfer indicating that the agent is capable of inhibiting fusion of HIV-1 to CD4$^+$ cells.

HIV-1 only fuses with appropriate CD4$^+$ cells. For example, laboratory-adapted T lymphotropic HIV-1 strains fuse with most CD4$^+$ human cells. Clinical HIV-1 isolates do not fuse with most transformed CD4$^+$ human cell lines but do fuse with human primary CD4$^+$ cells such as CD4$^+$ T lymphocytes and macrophages. Routine experiments may be easily performed to determine whether the CD4$^+$ cell is appropriate for the above fusion assay.

As described in this invention, HIV-1 membrane fusion is monitored by a resonance energy transfer assay. The assay was described in the International Application Number, PCT/US94/14561, filed December 16, 1994 with International Publication Number WO 95/16789. This assay is further elaborated in a United States co-pending application no. 08/475,515, filed June 7, 1995. The contents of these applications are hereby incorporated by reference into this application.

In an embodiment of the above method, the non-chemokine agent is an oligopeptide. In another embodiment, the non-chemokine agent is a polypeptide. In still another embodiment, the agent is an antibody or a portion thereof. In a further embodiment, the non-chemokine agent is a nonpeptidyl agent.

In a separate embodiment, the CD4$^+$ cell is a PM1 cell. In another embodiment, the cell expressing the HIV-1 envelope glycoprotein is a HeLa cell expressing HIV-1$_{JR-FL}$ gp120/gp41.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

FIRST SERIES OF EXPERIMENTS

1) Chemokines inhibit fusion mediated by the envelope glycoprotein from a macrophage-tropic primary isolate of HIV-1 but not from a laboratory-adapted T-lymphotrophic strain of the virus

The chemokines RANTES, MIP-1α and MIP-1β were obtained from R & D systems (Minneapolis, MN). They were tested in the RET assay for ability to inhibit fusion between HeLa-env$_{JR-FL}$ cells (expressing gp120/gp41 from the macrophage tropic isolate HIV-1$_{JR-FL}$) and PM1 cells, or for inhibition of fusion between HeLa-env$_{LAI}$ cells (expressing gp120/gp41 from the laboratory-adapted strain HIV-1$_{LAI}$) and various CD4$^+$ T lymphocyte cell lines. As shown in Figure 1, all three chemokines inhibited fusion mediated by the macrophage tropic virus envelope glycoprotein, but not that mediated by the laboratory-adapted strain envelope glycoprotein.

The ability of the chemokines to block the interaction between CD4 and HIV-1 gp120 which occurs at virus attachment was then tested. It was found that the chemokines did not inhibit this interaction (Figure 2), demonstrating that their blockade of HIV-1 envelope glycoprotein-mediated membrane fusion occurs at the membrane fusion event itself, rather than the initial CD4-gp120 interaction which precedes fusion.

2) Non-chemokine peptides and derivatives that inhibit HIV-1 fusion

The non-chemokines include chemokine fragments and chemokine derivatives that are tested in the RET assay to determine which are active in inhibiting HIV-1 membrane fusion. Particular attention is focused on fragments or derivatives

that inhibit HIV-1 fusion but do not activate leukocyte responses.   These non-chemokines include:

a) N-terminal derivatives of the chemokines.   Addition of
5   residues to the N-terminus of chemokines inhibits the function of these proteins without significantly reducing their ability to bind chemokine receptors.   For example, Met-RANTES (RANTES with an N-terminal methionine) has been shown to be a powerful antagonist of native RANTES and is
10   unable to induce chemotaxis or calcium mobilization in certain systems.   The mechanism of antagonism appears to be competition for receptor binding (9).   Similar results were found using other derivatives of the N terminus of RANTES(9) and also by N-terminal modification of other chemokines,
15   such as IL-8 (a member of the C-X-C chemokines) (10).   The current invention includes Met-RANTES and other chemokines derivatised by the addition of methionine, or other residues, to the N-terminus so that they inhibit fusion mediated by the envelope glycoprotein of HIV-1$_{JR-FL}$, and
20   inhibit infection by many isolates of HIV-1, yet do not activate the inflammatory response.

b)   Chemokines with N-terminal amino acids deleted: Chemokine antagonists have been generated by deleting amino
25   acids in the N-terminal region.   For example, deletion of up to 8 amino acids at the N-terminus of the chemokine MCP-1 (a member of the C-C chemokine group), ablated the bioactivity of the protein while allowing it to retain chemokine receptor binding and the ability to inhibit activity of
30   native MCP-1 (11,12).

The current invention includes N-terminal deletants of RANTES, MIP-1α and MIP-1β, lacking the biological activity of the native proteins, which inhibit HIV-1 fusion and HIV-1
35   infection.

c) Other peptides: A series of overlapping peptides (e.g. of
20-67 residues) from all regions of RANTES, MIP-1α and
MIP-1β are screened by the same approaches to identify
peptides which inhibit HIV-1 fusion most potently without
5   activating leukocytes. Activation of leukocyte responses is
measured following routine procedures (9, 10, 11, 12).

3)   Cloning the chemokine receptors

Chemokine receptors required for HIV-1 fusion are cloned by
the following strategy. First a cDNA library is made in a
10   mammalian expression vector (e.g. pcDNA3.1 from Invitrogen
Corp. San Diego, CA) using mRNA prepared from the PM1 cell
line or CD4⁺ T-lymphocytes or macrophages. Degenerate
oligonucleotide probes are used to identify members of the
cDNA library encoding members of the chemokine receptor
15   family, for example following previously published methods
(2). The vectors containing chemokine receptor cDNAs are
then individually expressed in one of several mammalian cell
lines which express human CD4 but do not fuse with
HeLa-env$_{JR-FL}$ cells (e.g. HeLa-CD4, CHO-CD4 or COS-CD4) or
20   HeLa-env$_{LAI}$ cells (e.g. CHO-CD4 or COS-CD4). Following
analysis in the RET assay, clones which gain the ability to
fuse with HeLa-env$_{JR-FL}$ or HeLa-env$_{LAI}$ are identified and the
coding sequences recovered, for example by PCR
amplification, following procedures well known to those
25   skilled in the art. DNA sequencing is then performed to
determine whether the cDNA recovered encodes a known
chemokine receptor. Following expression of the receptor,
monoclonal and polyclonal antibodies are prepared and tested
for ability to inhibit infection by a panel of HIV-1
30   isolates.

References of the First Series of Experiments

1. Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., Lusso, P. 1995. *Science*. 270:1811-1815.

2. Raport, C. J., Schweickart, V. L., Chantry, D., Eddy Jr., R. L., Shows, T. B., Godiska, R., Gray, P. W. 1996. *Journal of Leukocyte Biology*. 59: 18-23.

3. Maddon PJ., Dalgleish AG., McDougal JS., Clapham PR., Weiss RA., Axel R. 1986. *Cell*. 47:333-348.

4. Ashorn PA., Berger EA., Moss B. 1990. *J. Virol.* 64:2149-2156.

5. Clapham PR., Blanc D., Weiss RA. 1991. *Virology*. 181:703-715.

6. Harrington RD., Geballe AP. 1993. *J. Virol.* 67:5939-5947.

7. Broder CC., Dimitrov DS., Blumenthal R., Berger EA. 1993. *Virology*. 193:483-491.

8. Dragic T., Charneau P., Clavel F., Alizon M. 1992. *J. Virol.* 66:4794-4802.

9. Wells, T. N., Power, C. A., Lusti-Narasimhan, M., Hoogewerf, A. J., Cooke, R. M., Chung, C. W., Peitsch, M. C., Proudfoot, A. E. 1996. *Journal of Leukocyte Biology*. 59:53-60.

10. Moser, B., Dewald, B., Barella, L., Schumacher, C., Baggiolini, M., Clark-Lewis, I. 1993. *Journal of*

Biological Chemistry. 268:7125-7128.

11. Gong, J. H., Clark-Lewis, I. 1995. *J. Exp. Med.* 181:631-640.

12. Zhang, Y. J., Rutledge, B. J., Rollins, B. J. 1994. *Journal of Biological Chemistry.* 269:15918-15924.

13. Merrifield, R.B. (1963) *J. Am. Chem. Soc.* 85: 2149-2154.

14. Goeghegan, K.F. Stroh, J.F. (1992) *Bioconjugate Chem.* 3: 138-146.

SECOND SERIES OF EXPERIMENTS

The replication of primary, non-syncytium-inducing (NSI) HIV-1 isolates in CD4^{+} T-cells is inhibited by the C-C ß-chemokines MIP-1α, MIP-1ß and RANTES (1,2), but T-cell line-adapted (TCLA) or syncytium-inducing (SI) primary strains are insensitive (2,3). The ß-chemokines are small (8kDa), related proteins active on cells of the lymphoid and monocyte lineage (4-8). Their receptors are members of the 7-membrane-spanning, G-protein-linked superfamily, one of which (the LESTR orphan receptor) has been identified as the second receptor for TCLA HIV-1 strains, and is now designated fusin (9). Fusin is not known to be a ß-chemokine receptor (7-9).

To study how ß-chemokines inhibit HIV-1 replication, a virus entry assay based on single-cycle infection by an env-deficient virus, NL4/3Δenv (which also carries the luciferase reporter gene), complemented by envelope glycoproteins expressed in trans was used (10,11). Various env-complemented viruses were tested in PM1 cells, a variant of HUT-78 that has the unique ability to support replication of primary and TCLA HIV-1 strains, allowing comparison of envelope glycoprotein functions against a common cellular background (2,12). MIP-1α, MIP-1ß and RANTES are most active against HIV-1 in combination (2,3), and strongly inhibited infection of PM1 cells by complemented viruses whose envelopes are derived from the NSI primary strains ADA and BaL (Table 1a).

Table 1: Inhibition of HIV-1 entry in PM1 cells and CD4^{+} T-cells by ß-chemokines

% luciferase activity

	BaL	ADA	NL4/3	HxB2	MuLV
a)					
PM1 cells					
control without virus	2	2	2	5	3
control with virus	100	100	100	100	100
+R/Mα/Mß (50/50/50)	2	3	92	117	100
+RANTES (100)	1	1	nd	nd	nd
+MIP-1α(100)	54	54	nd	nd	nd
+MIP-1ß (100)	1	6	nd	nd	nd
+MCP-1 (100)	46	50	nd	nd	nd
+MCP-2 (100)	28	26	nd	nd	nd
+MCP-3 (100)	58	46	nd	nd	nd
b)	JR-FL	HxB2	MuLV		
LW4 CD4^{+} T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	14	68	nd		
LW5 CD4^{+} T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	15	73	nd		

Table 1 legend:

PM1 cells were cultured as described by Lusso et al (12). Ficoll/hypaque-isolated PBMC from laboratory workers (LW) stimulated with PHA for 72h before depletion of CD8+ Lymphocytes with anti-CD8 immunomagnetic beads (DYNAL, Great
5   Neck, NY). CD4+ Lymphocytes were maintained in culture medium containing interleukin-2 (100U/ml; Hofmann LaRoche, Nutley, NJ), as described previously (3). Target cells (1-2x10^{5}) were infected with supernatants (10-50ng of HIV-1 p24) from 293-cells co-transfected with an
10   NL4/3Δenv-luciferase vector and a HIV-1 env-expressing vector (10,11). ß-Chemokines (R & D Systems, Minneapolis) were added to the target cells simultaneously with virus, at the final concentrations (ng/ml) indicated in parentheses in the first column. The ß-chemokine concentration range was

selected based on prior studies (2,3). After 2h, the cells were washed twice with PBS, resuspended in ß-chemokine-containing media and maintained for 48-96h. Luciferase activity in cell lysates was measured as
5   described previously (10,11). The values indicated represent luciferase activity (cpm)/ng p24/mg protein, expressed relative to that in virus-control cultures lacking ß-chemokines (100%), and are the means of duplicate or sextuplicate determinations. nd, not done. R/Mα/Mß, RANTES
10   + MIP-1α + MIP-1ß.

RANTES and MIP-1ß were strongly active when added individually, while other ß-chemokines - MlP-1α, MCP-1, MCP-2 and MCP-3 (refs. 13-15) - were weaker inhibitors
15   (Table 1a). However, MIP-1α, MIP-1ß and RANTES, in combination, did not inhibit infection of PM1 cells by the TCLA strains NL4/3 and HxB2, or by the amphotropic murine leukemia virus (MuLV-Ampho) pseudotype (Table 1a). Thus, phenotypic characteristics of the HIV-1 envelope
20   glycoproteins influence their sensitivity to ß-chemokines in a virus entry assay.

The env-complementation assay was used to assess HIV-1 entry into CD4+ T-cells from two control individuals (LW4 and
25   LW5). MIP-1α, MIP-1ß and RANTES strongly inhibited infection by the NSI primary strain JR-FL infection of LW4's and LW5's CD4+ T-cells, and weakly reduced HxB2 infection of LW cells (Table 1b), suggesting that there may be some overlap in receptor usage on activated CD4+ T-cells by
30   different virus strains. BaL env-mediated replication in normal PBL was also inhibited by MIP-1α, MIP-1ß and RANTES, albeit with significant inter-donor variation in sensitivity (data not shown).

35   It was determined when ß-chemokines inhibited HIV-1

replication by showing that complete inhibition of infection of PM1 cells required the continuous presence of ß-chemokines for up to 5h after addition of ADA or BaL env-complemented virus (Fig.3a). Pre-treatment of the cells with ß-chemokines for 2h or 24h prior to infection had no inhibitory effect if the cells were subsequently washed before virus addition. Furthermore, adding ß-chemokines 2h after virus only minimally affected virus entry (Fig.3a). A PCR-based assay was next used to detect HIV-1 early DNA reverse transcripts in PM1 cells after 10h of infection; reverse transcription of ADA, but not of NL4/3, could not be detected in the presence of MIP-1ß and RANTES (Fig.3b). Thus, inhibition by ß-chemokines requires their presence during at least one of the early stages of HIV-1 replication: virus attachment, fusion and early reverse transcription.

As described in part in the First Series of Experiments, these sites of action were discriminated, first by testing whether ß-chemokines inhibited binding of JR-FL or BRU (LAI) gp120 to soluble CD4, or of tetrameric CD4-IgG2 binding to HeLa-JR-FL cells expressing oligomeric envelope glycoproteins (17). No inhibition by any of the ß-chemokines was found in either assay, whereas the OKT4a CD4-MAb was strongly inhibitory in both (Fig. 2 and data not shown). Thus, ß-chemokines inhibit a step after CD4 binding, when conformational changes in the envelope glycoproteins lead to fusion of the viral and cellular membranes (18). Cell-cell membrane fusion is also induced by the gp120-CD4 interaction, and can be monitored directly by resonance energy transfer (RET) between fluorescent dyes incorporated into cell membranes (17). In the RET assay, OKT4a completely inhibits membrane fusion of PM1 cells with HeLa cells expressing the envelope glycoproteins of either JR-FL (HeLa-JR-FL, the same cell line referred to above as HeLa-

env$_{\text{JR-FL}}$) or BRU (HeLa-BRU, the same cell line referred to above as HeLa-env$_{\text{LAI}}$), confirming the specificity of the process (17). RANTES, MIP-1ß (and to a lesser extent, MIP-1α) strongly inhibited membrane fusion of HeLa-JR-FL cells with PM1 cells, whereas fusion between PM1 cells and HeLa-BRU cells was insensitive to these ß-chemokines (Fig. 1 and Table 2a).

**Table 2:Effect of ß-chemokines on HIV-1 envelope
glycoprotein-mediated membrane fusion
measured using the RET assay**

	% Fusion	
	HeLa-JR-FL	HeLa-BRU
a) PM1 cells		
no chemokines	100	100
+R/Mα/Mß (80/400/100)	1	95
+RANTES (80)	8	100
+MIP-1α (400)	39	100
+MIP-1ß (100)	13	93
+MCP-1 (100)	99	98
+MCP-2 (100)	72	93
+MCP-3 (100)	98	99
b) LW5 CD4+ cells		
no chemokines	100	100
+R/Mα/Mß(106/533/133)	39	100
+RANTES (106)	65	95
+MIP-1α (533)	72	100
+MIP-1ß (133)	44	92
+OKT4A (3ug/ml)	0	0

Table 2 legend:

*CD4+ target cells (mitogen-activated CD4+ lymphocytes or PM1
cells) were labeled with octadecyl rhodamine (Molecular
Probes, Eugene, OR), and HeLa-JR-FL cells, HeLa-BRU cells
(or control HeLa cells, not shown) were labeled with
octadecyl fluorescein (Molecular Probes), overnight at 37°C.
Equal numbers of labeled target cells and env-expressing
cells were mixed in 96-well plates and ß-chemokines (or CD4
MAb OKT4a) were added at the final concentrations (ng/ml)
indicated in parentheses in the first column. Fluorescence
emission values were determined 4h after cell mixing (17).*

If cell fusion occurs, the dyes are closely associated in the conjoined membrane such that excitation of fluorescein at 450nm results in resonance energy transfer (RET) and emission by rhodamine at 590nm. Percentage fusion is defined as equal to 100 x [(Exp RET - Min RET) / (Max RET - Min RET)], where Max RET = %RET obtained when HeLa-Env and CD4⁺ cells are mixed, Exp RET = %RET obtained when HeLa-Env and CD4⁺ cells are mixed in the presence of fusion-inhibitory compounds, and Min RET = %RET obtained when HeLa cells (lacking HIV-1 envelope glycoproteins) and CD4⁺ cells are mixed. The %RET value is defined by a calculation described elsewhere(17), and each is the mean of triplicate determinations. These values were, for HeLa-JR-FL and HeLa-BRU cells respectively: PM1 cells 11.5%, 10.5%; LW5 CD4⁺ cells, 6.0%, 10.5%; R/Mα/Mß, RANTES + MIP-1α + MIP-1ß.

Similar results were obtained with primary CD4⁺ T-cells from LW5 (Table 2b), although higher concentrations of ß-chemokines were required to inhibit membrane fusion in the primary cells than in PM1 cells. Thus, the actions of the ß-chemokines are not restricted to the PM1 cell line. The RET assay demonstrates that ß-chemokines interfere with env-mediated membrane fusion.

The simplest explanation of these results is that the binding of certain ß-chemokines to their receptor(s) prevents, directly or otherwise, the fusion of HIV-1 with CD4⁺ T-cells. It has been known for a decade that HIV-1 requires a second receptor for entry into CD4⁺ cells (19-21). This function is supplied, for TCLA strains, by fusin (9). Several receptors for MIP-1α, MIP-1ß and RANTES have been identified (6,7), and ß-chemokines exhibit considerable cross-reactivity in receptor usage (4-8). However, C-C CKR-1 and, especially, C-C CKR-5 were identified as the most likely candidates, based on tissue

expression patterns and their abilities to bind MIP-1α, MIP-1ß and RANTES (4,7,8,15,22). C-C CKR-1, C-C CKR-5 and LESTR are each expressed at the mRNA level in PM1 cells and primary macrophages (data not shown). These and other ß-chemokine receptors were therefore PCR-amplified, cloned and expressed.

The expression of C-C CKR-5 in HeLa-CD4 (human), COS-CD4 (simian) and 3T3-CD4 (murine) cells rendered each of them readily infectible by the primary, NSI strains ADA and BaL in the env-complementation assay of HIV-1 entry (Table 3).

Table 3: C-C CKR-5 expression permits infection of CD4-expressing cells by primary, NSI HIV-1 strains

		pcDNA3.1	LESTR	CKR-1	CKR-2a	CKR-3	CKR-4	CKR-5	R/Mα/MB CKR-5
COS-CD4	ADA	798	456	600	816	516	534	153000	3210
	BaL	660	378	600	636	516	618	58800	756
	HxB2	5800	96700	5240	5070	5470	5620	4850	5000
HeLa-CD4	ADA	678	558	4500	912	558	600	310000	6336
	BaL	630	738	1800	654	516	636	104000	750
	HxB2	337000	nd	nd	nd	nd	nd	nd	356000
3T3-CD4	ADA	468	558	450	618	534	606	28400	1220
	BaL	606	738	660	738	534	558	11700	756
	HxB2	456	24800	618	672	732	606	618	606

Table 3 legend:

Chemokine receptor genes C-C CKR-1, C-C CKR-2a, C-C CKR-3, C-C CKR-4 and C-C CKR-5 have no introns (4-8,15,22) and were isolated by PCR performed directly on a human genomic DNA pool derived from the PBMC of seven healthy donors. Oligonucleotides overlapping the ATG and the stop codons and containing BamHI and XhoI restriction sites for directional cloning into the pcDNA3.1 expression vector (Invitrogen Inc.) were used. LESTR (also known as fusin or HUMSTR) (4,9,24) was cloned by PCR performed directly on cDNA derived from PM1 cells, using sequences derived from the NIH database. Listed below are the 5'and 3' primer pairs used in first (5-1 and 3-1) and second (5-2 and 3-2) round PCR amplification of the CKR genes directly from human genomic DNA, and of LESTR from PM1 cDNA. Only a single set of primers was used to amplify CKR-5.

LESTR: L/5-1 = AAG CTT GGA GAA CCA GCG GTT ACC ATG GAG GGG ATC (SEQ ID NO: 6);

L/5-2 = GTC TGA GTC TGA GTC AAG CTT GGA GAA CCA (SEQ ID NO: 7);

L/3-1 = CTC GAG CAT CTG TGT TAG CTG GAG TGA AAA CTT GAA GAC TC (SEQ ID NO: 8);

L/3-2 = GTC TGA GTC TGA GTC CTC GAG CAT CTG TGT (SEQ ID NO: 9);

CKR-1:C1/5-1 = AAG CTT CAG AGA GAA GCC GGG ATG GAA ACT CC (SEQ ID NO: 10);

C1/5-2 = GTC TGA GTC TGA GTC AAG CTT CAG AGA GAA (SEQ ID NO: 11);

C1/3-1 = CTC GAG CTG AGT CAG AAC CCA GCA GAG AGT TC (SEQ ID NO: 12);

C1/3-2 = GTC TGA GTC TGA GTC CTC GAG CTG AGT CAG (SEQ ID NO: 13);

CKR-2a:C2/5-1 = AAG CTT CAG TAC ATC CAC AAC ATG CTG TCC AC (SEQ ID NO: 14);

C2/5-2= *GTC TGA GTC TGA GTC AAG CTT CAG TAC ATC (SEQ ID NO: 15);*

C2/3-1 = *CTC GAG CCT CGT TTT ATA AAC CAG CCG AGA C (SEQ ID NO: 16);*

C2/3-2 = *GTC TGA GTC TGA GTC CTC GAG CCT CGT TTT (SEQ ID NO: 17);*

CKR-3: C3/5-1 = *AAG CTT CAG GGA GAA GTG AAA TGA CAA CC (SEQ ID NO: 18);*

C3/5-2= *GTC TGA GTC TGA GTC AAG CTT CAG GGA GAA (SEQ ID NO: 19);*

C3/3-1 = *CTC GAG CAG ACC TAA AAC ACA ATA GAG AGT TCC (SEQ ID NO: 20);*

C3/3-2 = *GTC TGA GTC TGA GTC CTC GAG CAG ACC TAA (SEQ ID NO: 21);*

CKR-4: C4/5-1 = *AAG CTT CTG TAG AGT TAA AAA ATG AAC CCC ACG G (SEQ ID NO: 22);*

C4/5-2 = *GTC TGA GTC TGA GTC AAG CTT CTG TAG AGT (SEQ ID NO: 23);*

C4/3-1 = *CTC GAG CCA TTT CAT TTT TCT ACA GGA CAG CAT C (SEQ ID NO: 24);*

C4/3-2 = *GTC TGA GTC TGA GTC CTC GAG CCA TTT CAT (SEQ ID NO: 25);*

CKR-5: C5/5-12 = *GTC TGA GTC TGA GTC AAG CTT AAC AAG ATG GAT TAT CAA (SEQ ID NO: 26);*

C5/3-12 = *GTC TGA GTC TGA GTC CTC GAG TCC GTG TCA CAA GCC CAC (SEQ ID NO: 37).*

The human CD4-expressing cell lines HeLa-CD4 (P42), 3T3-CD4 (sc6) and COS-CD4 (Z28T1) (23) were transfected with the different pcDNA3.1-CKR constructs by the calcium phosphate method, then infected 48h later with different reporter viruses (200ng of HIV-1 p24/10^6 cells) in the presence or absence of ß-chemokines (400ng/ml each of RANTES, MIP-1α and MIP-1ß). Luciferase activity in cell lysates was measured 48h later (10,11). ß-Chemokine blocking data is only shown for C-C CKR-5, as infection mediated by the other C-C CKR

genes was too weak for inhibition to be quantifiable. In PCR-based assays of HIV-1 entry, a low level of entry of NL4/3 and ADA into C-C CKR-1 expressing cells (data not shown) was consistently observed.

5

Neither LESTR nor C-C CKR-1, -2a, -3 or -4 could substitute for C-C CKR-5 in this assay. The expression of LESTR in COS-CD4 and 3T3-CD4 cells permitted HxB2 entry, and HxB2 readily entered untransfected (or control plasmid-transfected) HeLa-CD4 cells (Table 3). Entry of BAL and ADA into all three C-C CKR-5-expressing cell lines was almost completely inhibited by the combination of MIP-1α, MIP-1ß and RANTES, whereas HxB2 entry into LESTR-expressing cells was insensitive to ß chemokines (Table 3). These results suggest that C-C CKR-5 functions as a ß-chemokine-sensitive second receptor for primary, NSI HIV-1 strains.

The second receptor function of C-C CKR-5 was confirmed in assays of env-mediated membrane fusion. When C-C CKR-5 was transiently expressed in COS and HeLa cell lines that permanently expressed human CD4, both cell lines fused strongly with HeLa cells expressing the JR-FL envelope glycoproteins, whereas no fusion occurred when control plasmids were used (data not shown). Expression of LESTR instead of C-C CKR-5 did not permit either COS-CD4 or HeLa-CD4 cells to fuse with HeLa-JR-FL cells, but did allow fusion between COS-CD4 cells and HeLa-BRU cells (data not shown).

30

The fusion capacity of ß-chemokine receptors was also tested in the RET assay. The expression of C-C CKR-5, but not of C-C CKR-1, -2a, -3 or -4, permitted strong fusion between HeLa-CD4 cells and HeLa-JR-FL cells. The extent of fusion between HeLa-JR-FL cells and C-C CKR-5-expressing HeLa-CD4

cells was greater than the constitutive level of fusion between HeLa-BRU cells and HeLa-CD4 cells (Fig.4). The fusion-conferring function of C-C CKR-5 for primary, NSI HIV-1 strains has therefore been confirmed in two independent fusion assays.

Experimental Discussion

Together, the above results establish that MlP-1α, MIP-1ß and RANTES inhibit HIV-1 infection at the entry stage, by interfering with the virus-cell fusion reaction subsequent to CD4 binding. It was also shown that C-C CKR-5 can serve as a second receptor for entry of primary NSI strains of HIV-1 into CD4+ T-cells, and that the interaction of ß-chemokines with C-C CKR-5 inhibits the HIV-1 fusion reaction.

References of the Second Series of Experiments

1. Levy,J.A., Mackewicz,C.E. & Barker,E. Immunol. Today **17**, 217-224 (1996).

2. Cocchi,F. et al. Science **270**, 1811 -1815 (1995).

3. Paxton,W.A. et al. Nat. Med. **2**, 412-417 (1996).

4. Neote,K., DiGregorio,D., Mak,J.Y., Horuk,R., & Schall,T.J. Cell **72**, 415-425 (1993).

5. Gao,J.-L. et al. J . Exp. Med.**177**, 1421 -1427 (1993).

6. Bacon,K.B., Premack,B.A., Gardner,P. & Schall,T.J. Science **269**, 1727-1729 (1995).

7. Raport,C.J. etal. J. Leukoc. Biol. **59**,18-23 (1996).

8. Wells,T.N.C. et al. J. Leukoc. Biol. **59**, 53-60 (1996).

9. Feng,Y., Broder,C.C., Kennedy,P.E. & Berger,E.A. Science **272**, 872-877 (1996).

10. Chen,B.K., Saksela,K., Andino,R. & Baltimore,D. J. Virol. **68**, 654-660 (1994).

11. Connor,R.I., Chen,B.K., Choe,S., & Landau,N.R. Virology **206**, 935-944 (1995).

12. Lusso,P. etal. J. Virol. **69**, 3712-3720 (1995).

13. Charo,l.F. et al. Proc. Natl. Acad. Sci. USA **91**, 2752-2756 (1994).

14. Ben-Baruch,A. et al. J. Biol. Chem. **270**, 22123-22128 (1995).

15. Combadiere,C etal. J. Biol. Chem. **270**, 29671-29675 (1995).

16. Lip,J.P., D'Andrea,A.D., Lodish,H.F. & Baltimore,D. Nature **343**, 762-764 (1990).

17. Litwin,V. et al. J. Virol. (submitted for publication).

18. Moore,J.P., Jameson,B.A., Weiss,R.A. & Sattentau,Q.J. in Viral Fusion Mechanisms (ed Bentz,J.) 233-289 (CRC Press Inc, Boca Raton, USA,1993).

19. Maddon,P.J. et al. Cell **47**, 333-348 (1986).

20. Ashorn,P.A., Berger,E.A. & Moss,B. J.Virol. **64**, 2149-2156 (1990).

21. Clapham,P.R., Blanc,D. & Weiss,R.A. Virology **181**, 703-715 (1991).

22. Samson,M., Labbe,O., Mollereau,C., Vassart,G. & Parmentier,M. Biochemistry **11**, 3362-3367 (1996).

23. Dragic,T., Charneau,P., Clavel,F. & Alizon,M. J.Virol. **66**, 4794-4802 (1992)

24. Loetscher,M. et al. J.Biol.Chem. **269**, 232-237 (1994).

25. Moore,J.P. & Ho,D.D. AIDS **9 (suppl A),** S117-S136 (1995).

26. Trkola,A. & Moore,J.P. (unpublished data).

27. Chaudhuri,A., et al. 1994. J.Biol.Chem. **269**, 7835-7838 (1994).

28. Neote,K., Mak,J.Y., Kolakowski Jr.,L.F. & Schall,T.J. Blood **84**, 44-52 (1994).

29. Dragic,T., Picard,L. & Alizon,M. J.Virol. **69**, 1013-1018 (1995).

30. Puri,A., Morris,S.J., Jones,P., Ryan,M. & Blumenthal,R. Virology **219**, 262-267 (1996).31

THIRD SERIES OF EXPERIMENTS

The chemokine SDF-1 (stromal cell-derived factor 1) is the
natural ligand for Fusin/CXCR4 and blocks infection by
laboratory-adapted strains of HIV-1 (Ref. 1 and 2). SDF-1

5   exists as at least two forms, SDF-1α and SDF-1ß based on
variable splicing of the SDF-1 gene (Ref. 1 and 3) In the
RET assay, this chemokine specifically inhibits membrane
fusion mediated by gp120/gp41 form the laboratory-adapted
strain HIV_{LAI} but not by gp120/gp41 from the macrophage-

10   tropic isolate $HIV-1_{JR-FL}$ as shown in Figure 5.

References of the Third Series of Experiments

1.   Bleul, C.C., et al. (1996) *Nature* 382:829-833
2.   Oberlin, E., et al. (1996) *Nature* 382:833-835

15   3.   Shirozu, M., et al. (1995) *Genomics* 28:495-500

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANTS: Allaway, Graham P
                 Litwin, Virginia M
                 Maddon, Paul J
                 Olson, William C

    (ii) TITLE OF INVENTION: A Method For Preventing HIV-1 Infection of CD4+
                     Cells

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       (A) MEDIUM TYPE: Floppy disk
       (B) COMPUTER: IBM PC compatible
       (C) OPERATING SYSTEM: PC-DOS/MS-DOS
       (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

   (vi) CURRENT APPLICATION DATA:
       (A) APPLICATION NUMBER:
       (B) FILING DATE:
       (C) CLASSIFICATION:

  (viii) ATTORNEY/AGENT INFORMATION:
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       (B) REGISTRATION NUMBER: 28678
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       (B) TELEFAX: 212-391-0525

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 38 nucleotides
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: oligonucleotide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAGGCTACT TCCCTGATTG GCAGAACTAC ACACCAGG   38

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 25 nucleotides
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single

```
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCAAGCCGA GTCCTGCGTC GAGAG                                              25

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGACTTTCC GCTGGGGACT TTC                                                23

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCTGTTCGGG CGCCACTGCT AGAGATTTTC CAC                                     33

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 60 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: n/a
        (D) TOPOLOGY: n/a

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

    Pro Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys
    1               5                   10                  15

    Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe Val
            20                  25                  30

    Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp Val Arg
    35                  40                  45                  50

    Glu Tyr Ile Asn Ser Leu Glu Met Ser
                55                  60
```

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGCTTGGAG AACCAGCGGT TACCATGGAG GGGATC   36

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTCTGAGTCT GAGTCAAGCT TGGAGAACCA   30

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCGAGCATC TGTGTTAGCT GGAGTGAAAA CTTGAAGACT C   41

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTCTGAGTCT GAGTCCTCGA GCATCTGTGT   30

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single

```
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: oligonucleotide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGCTTCAGA GAGAAGCCGG GATGGAAACT CC                              32

(2) INFORMATION FOR SEQ ID NO:11:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: oligonucleotide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTCTGAGTCT GAGTCAAGCT TCAGAGAGAA                                 30

(2) INFORMATION FOR SEQ ID NO:12:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 32 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: oligonucleotide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTCGAGCTGA GTCAGAACCC AGCAGAGAGT TC                              32

(2) INFORMATION FOR SEQ ID NO:13:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: oligonucleotide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCTGAGTCT GAGTCCTCGA GCTGAGTCAG                                 30

(2) INFORMATION FOR SEQ ID NO:14:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 32 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: oligonucleotide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
```

AAGCTTCAGT ACATCCACAA CATGCTGTCC AC   32

(2) INFORMATION FOR SEQ ID NO:15:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: oligonucleotide

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTCTGAGTCT GAGTCAAGCT TCAGTACATC   30

(2) INFORMATION FOR SEQ ID NO:16:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 31 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: oligonucleotide

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCGAGCCTC GTTTTATAAA CCAGCCGAGA C   31

(2) INFORMATION FOR SEQ ID NO:17:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: oligonucleotide

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTCTGAGTCT GAGTCCTCGA GCCTCGTTTT   30

(2) INFORMATION FOR SEQ ID NO:18:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 29 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: oligonucleotide

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGCTTCAGG GAGAAGTGAA ATGACAACC   29

(2) INFORMATION FOR SEQ ID NO:19:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 nucleotides

```
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:
```

GTCTGAGTCT GAGTCAAGCT TCAGGGAGAA   30

(2) INFORMATION FOR SEQ ID NO:20:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
```

CTCGAGCAGA CCTAAAACAC AATAGAGAGT TCC   33

(2) INFORMATION FOR SEQ ID NO:21:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
```

GTCTGAGTCT GAGTCCTCGA GCAGACCTAA   30

(2) INFORMATION FOR SEQ ID NO:22:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:
```

AAGCTTCTGT AGAGTTAAAA AATGAACCCC ACGG   34

(2) INFORMATION FOR SEQ ID NO:23:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide
```

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTCTGAGTCT GAGTCAAGCT TCTGTAGAGT   30

(2) INFORMATION FOR SEQ ID NO:24:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 34 nucleotides
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: oligonucleotide

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTCGAGCCAT TTCATTTTTC TACAGGACAG CATC   34

(2) INFORMATION FOR SEQ ID NO:25:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 nucleotides
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: oligonucleotide

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTCTGAGTCT GAGTCCTCGA GCCATTTCAT   30

(2) INFORMATION FOR SEQ ID NO:26:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 39 nucleotides
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: oligonucleotide

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTCTGAGTCT GAGTCAAGCT TAACAAGATG GATTATCAA   39

(2) INFORMATION FOR SEQ ID NO:27:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 39 nucleotides
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: oligonucleotides

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTCTGAGTCT GAGTCCTCGA GTCCGTGTCA CAAGCCCAC   39